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Epigenome, Cancer Prevention and Flavonoids and Curcumin

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Additional information is available at the end of the chapter

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1. Introduction

Epigenome is a common name for heritable chemical modifications of DNA and histone molecules, of which DNA methylation and histone acetylation and methylation represent the most studied parts. Nucleosomes, the chromatin building units, are positioned in a way that is strictly dependent on the epigenome changes. Based on the presence of a specific epigenetic modification, the chromatin becomes less or more condensed. These changes in chromatin structure are inevitably related to gene activity. For example, DNA hypermethylation joined with histone hypoacetylation is frequently related to a condensed form of chromatin, marking the region of DNA that should not be active during a specific time window. This implies that genes in that specific region may become active once the aforementioned marks are removed. Indeed, epigenome represents a very powerful, extremely flexible “tool” for regulating gene activity and the major reason for the well-known phenomena of “time specific” and “tissue specific” gene expression.

In the field of cancer research, epigenome changes are considered to be among the first steps in carcinogenesis, preceding the structural changes in the DNA molecule, known generally as “gene mutations”. Specifically, the most prominent change in the earliest phases of cancer is inactivation of tumor suppressor genes which are frequently silenced through DNA methylation and histone deacetylation taking place in the regions corresponding to their promoters. It is known that enzymes regulating these processes, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) are aberrantly up-regulated not only in a developed cancer, but also in the early phases of carcinogenesis (as recently shown for ductal *in situ* breast cancer [1]). Accordingly, significant effort has been given to the discovery and development of specific chemical compounds that may act as DNA demethylating agents and histone deacetylases

inhibitors (HDACi). Some of these compounds are already utilized in the clinic, like DNMT inhibitors 5-azacytidine and decitabine and HDAC inhibitor vorinostat, with specific therapeutic indications [2]. In parallel with these research efforts, considerable data has been published on the role of natural compounds. The importance of these compounds has been intensely studied in the field of cancer prevention, especially in light of the influence of a specific diet on cancer prevention (as shown for the Mediterranean diet which may have a modest beneficial effect related to the incidence of colorectal carcinoma [3]). Many dietary compounds have been revealed to significantly impact gene expression through modulating the epigenome. Logically, if a specific type of diet is considered to be protective in relation to cancer, there has to be a molecular mechanism at the level of the epigenome explaining its protective effect.

These will be addressed in this book chapter, where we describe polyphenols' molecular mechanisms of action, through well-known compounds belonging to different chemical subgroups and shown in Table 1.

2. Chromatin structure

The native state of DNA, a double helix, is formed when two antiparallel strands are held together by hydrogen bonds (H-bonds) between complementary purine and pyrimidine bases. Each human diploid cell contains approximately two meters of DNA. Obviously, in order to be kept in the nucleus, which is, on average, nine micrometers in diameter, the DNA must be 10,000-20,000 folds compacted [4].

Histones are small proteins that act as chromatin "compacting units. The tight binding of DNA and histones is considerably dependent on the charge: DNA is negatively charged molecule due to presence of phosphate groups, and the strength of interaction between DNA and histones strongly depends on posttranslational modifications of histones' tails which can modify their charge. This is possible due to a specific structure of histone proteins. Histones' globular domain represents central part that is highly structured. In addition to this part, histone proteins have "tails" at both, NH₂- and COOH-ends. These tails may be modified through numerous types of posttranslational modifications of which methylation and acetylation will be discussed in detail through this chapter. These covalent modifications, by which cell regulates access to DNA, depend on enzymes often called "writers" and "erasers" [5].

2.1. Histone modifications and gene transcription

The part of the genome that contains genes that are dynamically transcribed must be shaped in an open, flexible way. Part of the chromatin that follows this rule is called euchromatin. Non-coding parts of genome, joined with those parts that contain inactive genes (e.g., in the region of centromeres and telomeres) do not need to follow significant reshape. This condensed, closed shape of chromatin is known as heterochromatin. So, how do histone modifications influence keeping these two forms of chromatin present in the cell and, also important, how do histone modifications influence "the switch" between the two forms? In other words,

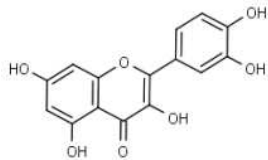
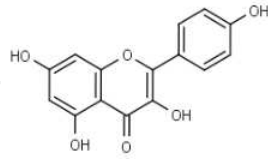
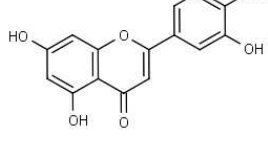
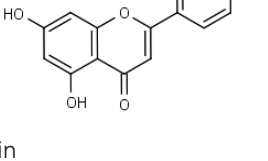
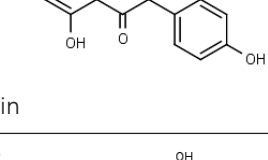
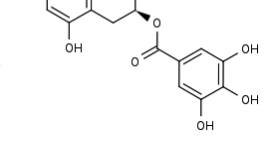
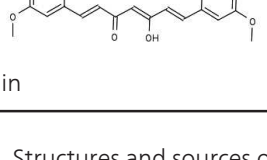
Polyphenol	Plant sources
	Apples, grapes, blackberries, cranberries, blueberries, onion, kale, capers, watercress, dill, bitter dock, red wines, black tea
Quercetin	
	Tea, kale, carrot, capers, leek, celery, apples
Kaempferol	
	Sage, thyme, peppermint, carrot, broccoli, onion, chilli
Luteolin	
	Chinese cabbage, parsley, papper, garlic , barley, endive, olive oil, chamomile, apples, onion
Apigenin	
	Soy, soy food – miso, tempeh, tofu etc.
Genistein	
	Green tea
EGCG	
	Turmeric (<i>Curcuma longa</i>), curry
Curcumin	

Table 1. Structures and sources of selected polyphenols with known epigenome modifying activities.

how is the need for expression of a specific gene during very short time period regulated by histone posttranslational modifications?

There are two basic mechanisms: a) direct, influencing the overall structure of chromatin, b) indirect, through the regulation of binding of effector molecules [6]. For example, trimethylation of lysine at the position 9 of histone 3 (H3K9me3) can recruit the protein HP1 (heterochromatin protein 1). The interaction takes place in the area of heterochromatin and leads to additional compacting that physically precludes access of transcriptional machinery [7]. There is sufficient level of evidence on *cis*-interactions in chromosome-mediated gene expression. For example, H3S10 phosphorylation enhances H3K4 methylation and H3K14 acetylation, and inhibits H3K9 methylation, thus indirectly facilitating chromatin decondensation [8].

The first report on histone acetylation is 50 years old [9]. The earliest studies following this discovery, revealed association of hyperacetylated histones with gene transcription [10]. In general, keeping in mind that there is no rule that would not have an exception, hyperacetylation of histone tails induces transcriptional activation, while hypoacetylation is associated with repression of transcription. Lysine acetylation is very dynamic process, regulated by antagonistic actions of two groups of enzymes: histone acetyltransferases (HATs) and HDACs. The consequence of lysine acetylation, which occurs upon the transfer of an acetyl group to the ϵ -amino-group of lysine side chain, is neutralization of lysine's positive charge and hence weakening interactions between histone and DNA. Mainly as a result of cumulative charge neutralizations at multiple lysines, the chromatin takes its "open", transcriptionally active structure [11]. This process is fast and happens in only a few minutes, as measured by isotopic pulse labeling [12]. In general, HDACs have the suppressing function, they are negative regulators of gene activity. It must be told that these enzymes have also many non-histone protein substrates that are included in regulation of important cellular processes, including cell proliferation and cell death. These are but not limited to: transcription factors, hormone receptors, cytoskeletal proteins, chaperons and various signaling mediators.

Histone methylation certainly is one of the best studied histone modifications, which takes place on both lysine and arginine amino acid residues. These modifications are not only residue-dependent (K4, K9, K27, K36, K79 in histone 3 (H3)) but also status-dependent (mono-, di- and trimethylation). In most scenarios, H3K4me2/3, H3K36me1/3 and H3K79me1/2 and H4K20me1 modifications are associated with activation of transcription, while H3K9me2/3, H3K27me2/3 and H4K20me3 marks are usually associated with transcriptional repression [13]. H3K4me3 association with 5' regions of active genes strongly correlates with overall transcription rate, active RNA polymerase II occupancy and global histone acetylation (reviewed in [14]). Such outcome is a consequence of binding of different proteins that specifically interact with activators or repressors of transcription.

Histone methylation depends on activity of histone methylating (histone lysine methyltransferases, KMTs) and demethylating (histone lysine demethylases, KDMs) enzymes. Both groups of enzymes have a very important role in cancer initiation and development and their mutations have been shown to exist in different types of malignant tumors. Methylation marks on histones are highly specific, altering gene expression in a way that may be unique for particular type of normal and/or cancer cell [13]. For example, histone demethylase KDM5A

contains a highly specific H3K4me3 reader domain and is probably involved in modifying nearby domains upon binding to H3K4me3. This enzyme was found to be present in regions that are enriched in H3K4me3 and are transcriptionally active. This is particularly significant in cells originating from bone marrow and peripheral blood where high expression of its target genes was also found [13]. For that reason, the ratio of various histone demethylases and histone methyltransferases, KDM/KMT, was suggested to be a sign of a specific "transcription module", trying to predict expression of specific genes, members of specific module. This fact, which should provide more specific way of acting compared to acetylation balancing enzymes, makes enzymes involved in regulation of histone methylation status good candidates for specific epigenetic interventions.

Histone lysine methylation depends on KMTs that are classified into two groups, based on the sequence and structure of their catalytic domains: SET domain containing (suppressor of variegation homologous (SUV), enhancer of zeste proteins (EZH), trithorax-group (TrxG) proteins) and non-SET domain containing (DOT1 protein family) [15].

So far, histone demethylases KDMs are grouped in two families. KDM1 family includes only two members, LSD1 (lysine-specific demethylase 1, KDM1A) and LSD2 (KDM1B). LSD1 was the first discovered histone demethylase and its discovery revealed that histone lysine methylation is reversible process [16]. LSD1 targets histone H3K4, and, when associated with androgen or estrogen receptor, it can also target histone H3K9. In addition, LSD1 has been shown to demethylate non-histone proteins, including tumor suppressor p53 and DNA methyltransferase-1 (DNMT1). The second, Jumonji C (JmjC) domain containing protein family, is numerous and includes seven subgroups with a total of 14 KDMs. There is no doubt that KDMs and KMTs must work coordinately in order to keep a precise regulation of histone methylation.

Both KDMs and KMTs are involved in cancer initiation and progression, although KDMs are much less studied. There are firm data on histone lysine methyltransferases expression change upon cell exposure to heavy metals. For example, peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of healthy volunteers and exposed to varying concentration of nickel chloride (0.25, 0.5 and 1.0 mM) for 24 h and compared to untreated control samples. The transcriptome analyses (Affymetrix Human Genome U133.20 Array) combined with ChipSeq with anti-H3K4me3, have revealed that there are 1381 entities with 2-fold difference in expression upon nickel exposure. This number was reduced to 382 and 246 with more stringent cuts off - 3-fold and 5-fold, respectively. The global level of H3K4me3 was increased. This study also showed that H3K4me3 does not occupy only the promoters of genes that were transcriptionally active, but also extends into their coding regions, as previously shown for nickel-exposed human lung adenocarcinoma cell line A549 [17]. When measured in PBMC from subjects that had occupational exposure to nickel but did not have cancer, the level of H3K4me3 was significantly increased when compared with PBMC from subjects without exposure [17]. Since chronic exposure to nickel has been associated with lung and nasal carcinoma, these results, obtained on human population, clearly show that harmful exposure indeed reshapes chromatin in a direction that leads to carcinogenesis. So, if detected

early, before disease onset, can these changes be reversible? Even more so, can we influence the enzymes that reshape epigenome in a procarcinogenic direction?

2.2. Interplay between DNA methylation and histone acetylation

There are several chromatin-regulating factors that recognize methylated DNA or modified histone proteins. One of them, the HP1 protein, has already been mentioned. The common characteristic of these effector molecules is that they use a range of different recognition domains such as methyl-CpG-binding domains (MBD), zinc fingers (ZnF), chromo-domain, or plant homeodomain (PHD) in order to direct establishing specific epigenomic marks and orchestrate biological events [18, 19]. However, the most of studies performed so far have been conducted using isolated DNA or histone peptides and cannot recapitulate the situation found in chromatin *in vivo*. Recently, an approach named SNAP (SILAC nucleosome affinity purification) was used for the identification of proteins that are influenced by DNA CpG methylation and histone H3 K4-, K9- or K27-methylation (or a combination thereof) in the context of a nucleosome. The approach itself seems to be very valuable in defining the chromatin "interactome" and showing that DNA methylation and histone modifications act in a concerted manner by creating a "modification landscape" that must be interpreted by proteins able to recognize large molecular assemblies [20].

This is not surprising due to the fact that there are currently 18 HDACs identified in humans, subclassified in four classes: a) class I HDACs 1, 2, 3 and 8 localized mainly in nucleus where they acetylate numerous proteins, of which p53 (cellular tumor antigen p53) and BRCA1 (breast cancer type 1 susceptibility protein) are probably the most frequently studied non-histonic substrates; b) class II with class IIa of HDAC 4, 5, 7 and 9 isoforms located in nucleus and cytoplasm and class IIb of HDACs 6 and 10 located only in the nucleus; c) class III of sirutins SIRT1-7 whose activity depends on the presence of nicotinamide adenine dinucleotide (NAD⁺/NADH) and d) class IV containing only one enzyme, HDAC11 [21].

Besides actions on histone molecules, the importance of modifying "the guardian of genome" p53 represents utmost important cellular event. One of the consequences of recruitment of HDAC1 to p53, through MDM2 (E3 ubiquitin-protein ligase), is p53 deacetylation and degradation. For this very reason, there is no p53-related induction of p21^{WAF1} (p21, cyclin-dependent kinase inhibitor 1) and MDM2 [22]. Luckily, p21 is not regulated only by p53, but also through many other transcription factors including Sp1 (transcription factor Sp1) for which six binding sites are present in the p53 promoter. Thus, this is only one example of multiple regulations and how complex the system has to be in keeping the balance of signals that will prevent cell from uncontrolled division, which is a hallmark of cancer.

HDACi are generally subdivided into several groups, based on their diverse chemical structures-short chain fatty acids, hydroxamates, cyclic peptides, aliphatic acids, benzamides, SIRT inhibitors, electrophilic ketones and miscellaneous compounds [23]. The member of the hydroxamate group, suberoylanilide hydroxamic acid (SAHA, vorinostat) has been approved by FDA in October of 2006 for treatment of a rare cutaneous T-lymphoma. The approval for the cyclopeptide, romidepsin (Istodax®), came in November of 2009. Although well tolerated and with manageable side effects, HDACis have not entirely fulfilled expectations that were based on well-known molecular mechanism associated with their application *in vitro*: early

studies have undoubtedly shown that these HDACis upregulate p21 in p53-independent fashion leading to G2/M cell cycle arrest [24]. Only two years later it was shown that this treatment additionally increases cyclin E and decreases cyclin D1 [25].

As expected, the problem lies in insufficient selectivity of HDACis: in addition to an increase of p21 and other pro-apoptotic genes, the treatment with HDACis also induces transcription of genes that are pro-carcinogenic, like multiple isoforms of protein kinase C (PKC) and downstream regulated matrix metalloproteinases (MMPs). Among 30 cancer cell lines (12 liver, 10 lung, 5 gastric and 3 breast cancer cell lines) that were tested for cell migration (indicating metastatic potential) after being treated with HDACis belonging to different groups (including vorinostat), 43% (13/30) cell lines showed several hundred folds increase in migration potential [26]. This effect was initiated at low, non-cytotoxic sub- μ M to low μ M dosage of HDACis. In an *in vivo* experiment, tumor cell lines were injected into HDACi treated mice. In contrast to untreated control animals, these mice have developed metastatic disease with statistically proved significance ($p < 0.026$). However, when combined with curcumin or tamoxifen, this treatment had very promising effect both, *in vitro* and *in vivo*. Western blot analyses performed on tumor lysates obtained from mice treated with HDACi trichostatin A, combined with curcumin or tamoxifen revealed significant downregulation of PKCs and some other proteins included in tumor progression. Of importance, the high expression of p21 induced with HDACis remained unchanged.

It is well-known that dietary and other environmental factors induce epigenetic alterations. For example, smoking induces numerous harmful epigenetic alterations that can be first steps in malignant transformation. Accordingly, cellular epigenome may be protected from harmful events through a proper diet and healthy way of living.

With development of sophisticated methods applicable in all fields of molecular biology, anticancer properties have become proved for many natural compounds [27]. Their mode of action frequently influences cellular epigenome through chromatin remodeling [28]. For example, polyphenols can modulate chromatin structure through influencing expression of chromatin remodeling enzymes, HDACs and DNMTs [29]. Targeting these proteins in addition to various kinases (e.g. mitogen-activated protein kinases (MAPKs), phosphatidylinositolide-3-kinase (PI3K), protein kinase B isozymes (AKT), Aurora B, Polo-like kinase 1 (PLK1)), poly(ADP-ribose) polymerase (PARP) and other epigenome modifying enzymes KDMs (LSD1, JMJD3) and HATs (p300/CBP (CREB binding protein) coactivators) [30] make them extremely powerful modifiers of cellular processes included in all aspects of cellular survival and cellular death. Besides, for all these multilevel ways of acting, affecting a large number of biochemical signaling pathways, natural polyphenols are excellent examples of polypharmacological compounds.

3. Intracellular stability and nuclear localization of polyphenols

In order to exert their effect on chromatin organization, polyphenols must be stable and able to enter cellular nucleus. There are numerous studies in the field of cancer research reporting

on various changes in experiment-related pre-selected set of interesting proteins included in cellular proliferation, after adding a polyphenol to cellular medium. However, their intracellular accumulation, stability and transformation are poorly understood. This is not surprising, as there are only limited number of studies dealing with polyphenol's cellular uptake and efflux, their subcellular localization and intracellular stability [31]. These parameters are crucial for understanding their mechanism of action and biological effects.

The polyphenols are usually assumed to bind to cellular membrane and/or are in the cytoplasm affecting membrane receptors and modulating kinases activity [32,33]. Polyphenols whose effects on chromatin are described in this review, may cross both cellular and nuclear membranes and enter nucleus. For example, quercetin has been shown to be localized mainly in the nucleus of HepG2 cells [34,35]. Flavon apigenin has been reported to localize mostly in nuclear matrix and less in cytosol, nuclear membranes and microsomes of prostate cancer cell lines DU145, LNCaP and PC-3 [36]. By using a confocal laser scanning fluorescence microscope, the autofluorescence of galangin and kaempferol appeared stronger in the nucleus than cytoplasm of mouse hepatoma Hepa-1c1c7 cells [37]. Among flavonoids, only flavonols containing the conjugated 3-OH group (Figure 1), show autofluorescence and can be detected by this approach. The highest percentage of curcumin in breast cancer cell line MCF-7 cells has been found in membrane and decreases in the following order: membrane > cytoplasm > nucleus > mitochondria [38]. The fluorescent conjugates of EGCG were localized onto the membrane as well as in the cytoplasm and nucleus of mouse fibroblasts L-929 [39], but are found on the membrane, in the cytoplasm and specifically in mitochondria of the human umbilical vein endothelial cells (HUVECs) [40]. The largest fraction of soy isoflavone genistein has been localized in cytoplasm, but it has also been registered in nuclei and mitochondria of pooled female rat liver [41]. The associated concentrations of genistein were in the nmol/g range, far below the concentrations required for most of its *in vitro* effects [41]. Concentrations of parent polyphenols within cells *in vitro* are usually in low μM to sub- μM range [42].

Namely, significant portion of a polyphenol, such as in the cases of EGCG [43] and apigenin [44], may be intracellularly transformed, depending on cell type and cell culture conditions, particularly pH of the medium [43, 45]. At the cellular level, metabolic transformations of polyphenols through phases I and II enzymes, have been well-documented, including hydroxylation by CYP450 isoforms CYP1A2, CYP3A4 [44] and CYP1B1 [46] and methylation of hydroxyl groups by catechol-O-methyl transferase (COMT) [31]. These reactions may be necessary for generation of active metabolites, as is the case with conversion of apigenin into its main metabolite, luteolin [47]. Furthermore, since polyphenols more or less readily participate in redox processes, the contribution of their oxidized quinone/quinone methide forms to their mechanisms of action may not be excluded [48], particularly when relatively high concentrations of flavonoids (high μM to mM) have been applied to cells [31].

Metabolic conjugation alters the physicochemical properties of flavonoids and it is widely assumed that this can affect their biological activity. Hence, for understanding the cellular effects of parent polyphenol compounds, the influence of their uptake, intracellular metabolism, oxidation and localization should be taken into account or *a priori* excluded [49]. This is enormously important when put in the context of cellular epigenome which is specific for each

type of cell. It may not be surprising then that specific effects of metabolites are consistent with findings that polyphenols' actions are cell-type dependent.

As usual, observations presented in this review will be interpreted in terms of parent polyphenol aglycons whose structures are shown within Table 1, but one should bear in mind that they may be exerted by their metabolites and oxidized forms.

4. Dose-dependent effects of polyphenols

The cellular effects of polyphenols recorded *in vitro* are concentration- and time-dependent [49,50]. Application of quercetin and EGCG as well as other polyphenols like resveratrol, induces hormetic dose-response cellular phenomenon characterized by a low-dose stimulation and high-dose inhibition, often resulting in typical U-or J-shaped curves [50]. While showing pro-oxidant properties joined with cytotoxicity at high concentrations, low doses of quercetin (1-40 μ M) were shown to have antioxidant and chemopreventive properties [51]. Also, application of curcumin, even at a very low concentration (10 μ M), and especially if exposure was prolonged (up to 72 h), was shown to be cytotoxic for various cancer cells, but not for fibroblasts WI-38 [52].

At the low μ M doses, the polyphenols activate hormetic adaptive cellular stress response signaling pathways that include various kinases and transcription factors [50]. As a result, there is an activation of genes that encode stress resistance proteins such as antioxidant and detoxifying enzymes, protein chaperones, neurotrophic factors, and other cytoprotective proteins. Specific example of such a pathway is the Nrf2/ARE (NF-E2-related factor 2 / antioxidant response element) pathway [50]. At these concentrations, these compounds are not cytotoxic, but cytostatic. They commonly block the cell cycle in various cancer cells in S/G2 and mitotic phase, leading to cell death. They also target multiple kinases that are implicated in pathogenesis of cancer.

5. Plant polyphenols in cellular growth arrest and death

So far, all studied polyphenols have been shown to induce both, apoptosis and autophagy of cancer cells, without affecting normal cells [53,54,55]. Epicatechin and quercetin have been shown to stimulate autophagy by simultaneous promotion of cytoplasmic protein deacetylation although with different potencies and, possibly, through distinct molecular pathways [53]. Selected polyphenols can induce G0/G1 (growth arrest effect) and / or G2/S (anti-mitotic effect) phase cell cycle arrest of various human cancer cell lines [56]. The induction of autophagy and apoptosis is a very important mechanism for cancer prevention by dietary polyphenols. It represents an effective anti-cancer strategy since it is based on an effort to block or suppress the multistage process of carcinogenesis which relates to six fundamental hallmarks of cancer: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, maintenance of vascularization, and, finally, tissue invasion

and metastasis [57,58]. In addition, chemoprevention based on dietary polyphenols is not expected to be accompanied by side effects since they are selectively toxic for the cancer cells.

Mechanistically, polyphenols have been shown to induce growth arrest and increase apoptosis in cancer cells in various ways. Antiproliferative effects of dietary polyphenols are generally associated with their capacity to regulate response to oxidative stress and/or DNA damage, suppress angiogenesis, and inhibit various pro-proliferative signaling pathways. They affect all aspects of carcinogenesis at its different stages through inhibition of various survival transduction pathways related to NF- κ B, PI3K, AKT and MAP kinases, as well as by stimulating expression of tumor suppressor genes (p53, BRCA1 and BRCA2) [57,58]. All polyphenols studied so far, except apigenin, are able to modify redox cellular state as potent radical scavengers [59]. They are also direct inhibitors of enzymes included in production of reactive endogenous radicals. In addition to the effect on their enzymatic activity, dietary polyphenols are also modulators of expression of proteins related to cellular antioxidant defenses and detoxification, often through regulation of transcriptional activity of Nrf2.

These multiple effects of dietary polyphenols have been reviewed many times so far. Here, we are focused on reviewing recent studies of some polyphenols' influence on alteration of gene expression through modulating epigenome by affecting DNA methylation and posttranslational modifications of histone proteins. Natural compounds seem to offer a huge source of epigenetically active compounds of which flavonoids - flavonols quercetin and kaempferol, flavones apigenin and luteolin, isoflavone genistein, catechin EGCG and curcuminoid curcumin certainly take a very important place.

6. Quercetin

Quercetin is strong natural antioxidant flavonol ubiquitously present in dietary plant sources (Table 1) [59]. Cellular sensitivity and specific response to quercetin depends on sensitivity to hydrogen peroxide, H_2O_2 . For example, quercetin induces formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, in HL-60 cells, while in their H_2O_2 -resistant clone, HP 100 cells, this effect does not occur [60]. This, again, shows that mode of action of specific nutraceuticals depends on cellular "molecular background".

In addition to its substantial redox activity, quercetin has multiple intracellular molecular targets, affecting different signaling processes that are altered in cancer cells, with limited or no toxicity against normal cells. Accordingly, quercetin inhibits carcinogenesis primarily through inhibition of pro-proliferative signaling pathways. It has been found to be a pleiotropic kinase inhibitor [61]. At 2 μ M concentration, quercetin was shown to decrease the activity of 16 recombinant kinases by more than 80%, including ABL1, Aurora-A,-B,-C, CLK1, FLT3, JAK3, MET, NEK4, NEK9, PAK3, PIM1, RET, FGF-R2, PDGF-R α and -R β [62]. Many of these kinases are positive regulators of cell cycle. Thus, through simultaneous targeting of multiple pathways related to these kinases, quercetin may act as antiproliferative agent [61].

Part of quercetin's effects has been attributed to its ability to influence histone acetylation (Figure 1). Quercetin was reported to block the binding of different transactivators including

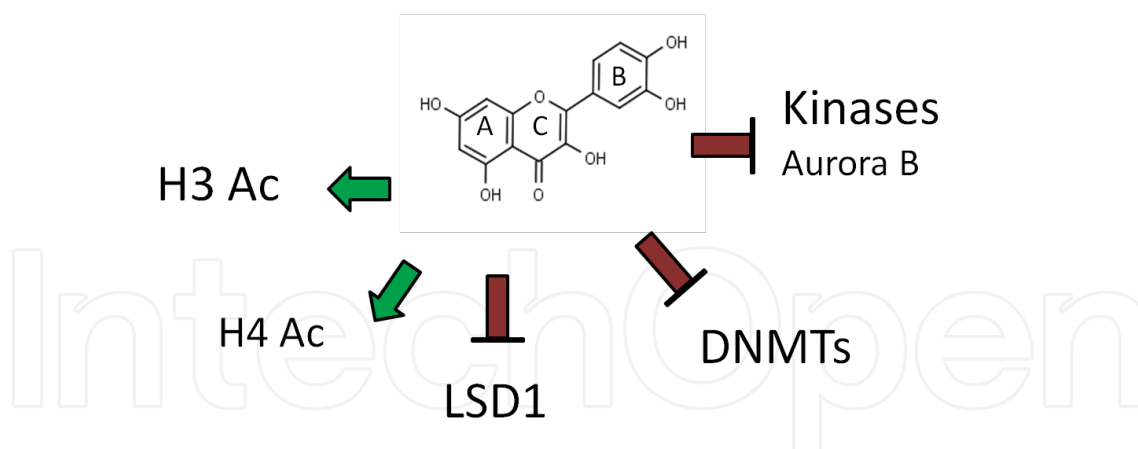


Figure 1. Epigenetic factors modulated by quercetin. Ring and atom annotations of 2-phenyl-1,4-benzopyrone scaffold.

p300, CREB2, c-Jun, C/EBP β and NF- κ B to the promoter of proinflammatory gene, COX2 (cyclooxygenase-2) [63]. As a consequence, there is a decrease in the level of the COX-2 protein. Generally, COX-2 decrease is considered to be a very beneficial step to a successful cancer chemoprevention [63,64]. While there is no doubt that quercetin affects numerous signaling pathways, one of these was well-explained on mouse intestinal epithelial cell line MODE-K, through its inhibitory effect on Akt phosphorylation, but without affecting cytoplasmic IKK (inhibitor of nuclear factor kappa-B (I κ B) kinase) activity. Thus, in this experimental model, quercetin had no direct effect on NF- κ B activation. Instead, it inhibited recruitment of the NF- κ B cofactor p300/CBP to the promoters of inflammatory genes IP-10 (10 kDa interferon gamma-induced protein, C-X-C motif chemokine 10) and MIP-2 (WD repeat-containing protein 26), through modulation of histone H3 acetylation and phosphorylation [65]. At 5 μ M concentration, quercetin was reported to increase acetylation of histones H3 and H4 induced by trichostatin A in A549 (expressing wild-type p53) and H1299 (a p53 null mutant) lung cancer cell lines, through p53 independent pathway [66].

Quercetin has also been found to effect activity of class III histone deacetylases SIRT1s. Due to the fact that biological effects of polyphenols depend on their own chemical stability and metabolism, it is sometimes hard to predict whether *in vitro* test results may precisely predict the situation in the living organism, *in vivo*. For example, it has been observed that quercetin increases deacetylase activity of recombinant SIRT1, but at cellular level it inhibits SIRT1 activity [67]. This has been explained by metabolic transformation of quercetin. When quercetin's metabolite, quercetin-3-O-glucuronide, was taken instead of quercetin, an inhibitory function on recombinant SIRT-1 was shown. However, quercetin activates SIRT1 deacetylase activity in hypoxia-exposed or hypoxic preconditioned HepG2 cells similarly to resveratrol, resulting in hypoxic down-regulation of *c-Myc* and β -catenin [68].

Sirtuins affect the acetylation status of histones as well as other important cellular non-histone proteins like HIF-1 α , c-Myc and β -catenin [68]. SIRT1-mediated histone deacetylation (H1K26, H3K9, H3K14, H4K16) is associated with the formation of heterochromatin. On the other hand, SIRT1 interacts with numerous transcription factors and, through modifying their acetylation

status, modifies their function. For example, in the presence of stress stimuli, SIRT1 deacetylates p53 and FOXO3 (forkhead box protein O3) leading to inhibition of apoptosis [69]. Another mechanism for inhibition of stress-induced apoptosis is through deacetylation of repair factor Ku70 (X-ray repair cross-complementing protein 6) and consequential sequestration of the proapoptotic factor BAX (Bcl-2-associated X protein) away from mitochondria [70]. While SIRT1 is capable of protecting cells from p53-induced apoptosis, its activity augments apoptosis in response to TNF- α (tumor necrosis factor alpha) by deacetylating RelA/p65 at lysine 310 and thus suppressing transcription of NF- κ B regulated genes [71].

Quercetin has been found to affect other class III HDAC, SIRT6, *in vitro*. Using frontal affinity chromatographic techniques, it has been found to inhibit deacetylase activity of immobilized recombinant SIRT6 protein against H3K9 [72].

Quercetin effects have been mostly studied on blood cancers. It induces apoptosis and autophagy of human leukemia HL-60 cells through orchestrating various signaling pathways and chromatin remodeling [73]. It has been reported to induce significant histone hyperacetylation at concentrations 75 and 100 μ M in human leukemia cells, indicating the possible involvement of histone hyperacetylation in its *in vitro* anticancer activity [73,74].

Quercetin has also been found to influence DNA as well as protein methylation levels. Hypermethylation of the tumor suppressor gene *p16^{INK4a}* (p16, cyclin-dependent kinase inhibitor 2A) in human colon cancer cell line RKO was successfully reversed after 120 h of treatment with quercetin [75]. This effect was concentration dependent.

Quercetin inhibiting activity against demethylase LSD1 was shown recently [76]. LSD1, commonly upregulated in cancer, has critical role in controlling the transcription of genes involved in cell growth and differentiation [77], and its inhibition may have significant therapeutic value. LSD1 functions primarily in multiprotein complexes, which includes, for example, HDAC1/2 and SIRT1.

Quercetin, as well as other catechol polyphenols, may indirectly inhibit DNMTs and thus DNA methylation, through changing intracellular concentrations of S-adenosyl methionine (SAM) and S-adenosyl-L-homocysteine (SAH) in a manner illustrated in Figure 2 [78].

7. Kaempferol

Kaempferol is a flavonol found in various vegetables (Table 1). In comparison with quercetin, it has considerably lower antioxidant capacity due to missing catechol moiety [59]. It possesses only 4'-OH group at ring B (Figure 1). However, like quercetin, kaempferol has a wide range of pharmacological activities, including antioxidant, anti-inflammatory and anticancer activity [79]. It suppresses growth of a number of human cancer cell lines in a concentration-dependent manner, through inducing an arrest in G0/G1 or G2/M phases of cell cycle [80]. Depending on the type of the cell, kaempferol can induce apoptosis [81,82] and autophagy [80]. For example, it induces apoptosis of human breast cancer cells MCF-7, by initiation of intrinsic caspase

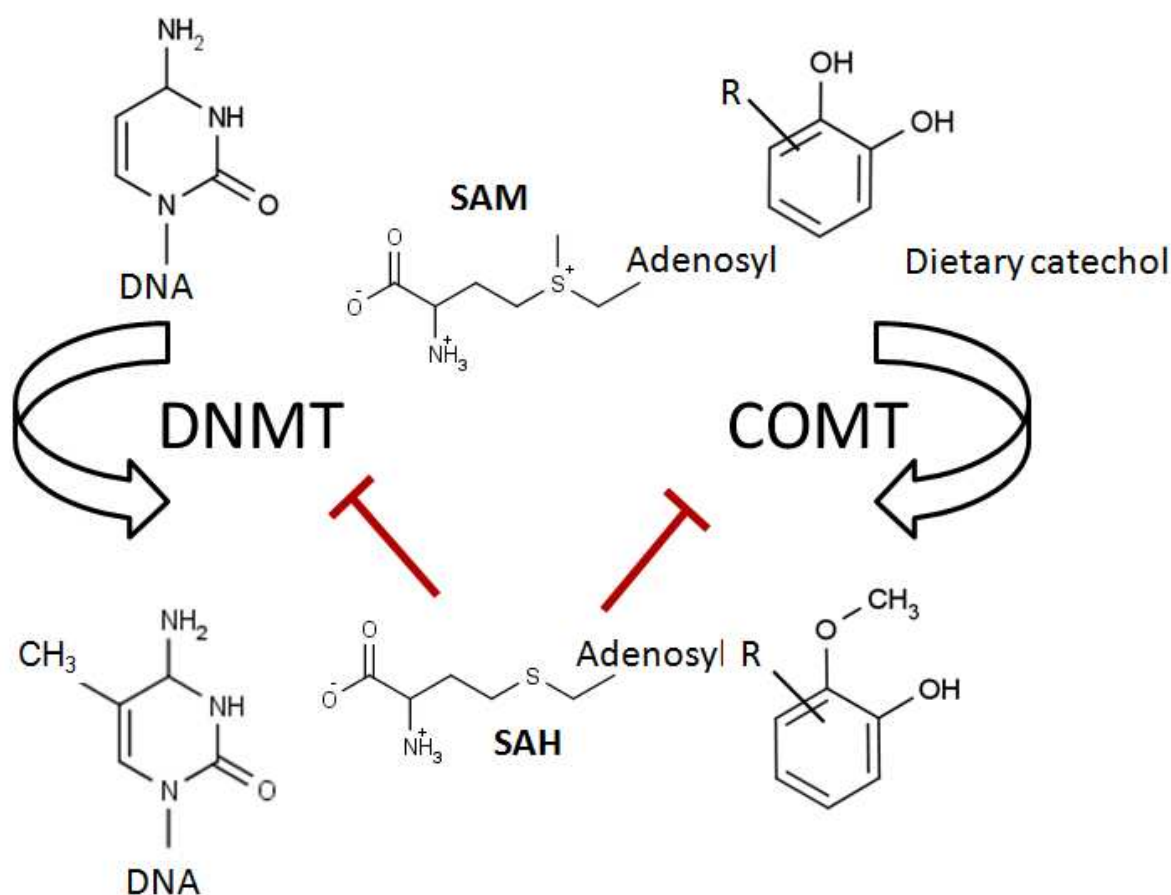


Figure 2. Indirect inhibitory effect of polyphenols with catechol (quercetin, luteolin) or galloyl (EGCG) moiety on DNMTs activity. In the O-methylation process of these polyphenols catalysed by the enzyme COMT, DNMT co-factor SAM is transformed into SAH which inhibits DNMTs and COMT.

cascade and down-regulation of the expression of nuclear kinase PLK1, an early trigger for G2/M transition and mitotic progression [83].

In human liver and colon cancer cell lines (HepG2, Hep3B, HCT-116) kaempferol (5-100 μ M) was shown to induce a prominent reduction of cell viability and proliferation rate, partly mediated through induction of hyperacetylation of histone H3 complex [81]. By performing *in silico* molecular docking analysis and *in vitro* profiling on these cancer cell lines, kaempferol has been recognized as a pan-inhibitor of human HDACs of classes I, II and IV [81].

Treatment of the chronic myelogenous leukemia cell line K562 and promyelocytic human leukemia U937 with 50 μ M kaempferol resulted in inhibition of PI3K and dephosphorylation of Akt at Ser473 and Thr308 as well as in an increase of the expression of deacetylase SIRT3 and its mitochondrial localization [84]. Finally, apoptotic death of these cells upon kaempferol exposure was a consequence of the induction of mitochondrial phase of the apoptotic program with an aforementioned increase of SIRT3, joined with an increase of BAX, decrease of Bcl-2, release of cytochrome c, and caspase-3 activation. Kaempferol was also shown to be an inhibitor of recombinant SIRT6 activity [72].

Similar to quercetin, kaempferol has shown inhibitory effect on histone demethylase LSD1 [76]. This enzyme has been reported to inversely regulate expressions of iodothyronine deiodinases of type 2 (D2) and type 3 (D3), acting as a molecular switch that dynamically finely tunes cellular needs for active thyroid hormone during myogenesis [85]. Kaempferol has been known to interfere with many aspects of the thyroid hormones synthesis [86]. LSD1 relieves the repressive marks (H3K9me_{2/3}) on the *DIO2* promoter and the activation marks (H3K4me_{2/3}) on the *DIO3* promoter. This action is well-orchestrated and depends on LSD1 interaction with transcription factor FoxO3. When FoxO3 binding to DNA is abrogated, LSD1 cannot induce D2.

8. Luteolin

Luteolin is a flavone widely distributed in the plant kingdom (Table 1). In difference to quercetin, it misses conjugated 3-OH group in C-ring (Figure 1) which considerably contributes to high radical scavenging capacity of quercetin [87]. Accordingly, luteolin is less efficient radical scavenger than quercetin [59]. However, the rest of structures of luteolin and quercetin are similar and hence similar biological activities have been observed. Both flavonoids have 3', 4'-catechol moiety in B-ring, resorcinol-like A-ring and extended π -electron delocalisation through whole skeleton. Luteolin inhibits growth of various cancer cell lines in a concentration-dependent manner [88]. The most common effect induced with luteolin, as well as other studied polyphenols with resorcinol-like structure of A-ring, is G2/M arrest of the cell cycle [88]. However, underlying mechanisms of action vary among flavonoid family members, primarily due to different number and positions of other hydroxyl groups at a common flavone (2-phenyl-1,4-benzopyrone) backbone, leading e.g. to different antioxidant capacities and intracellular metabolic transformations. The difference in antioxidative capacity of considered flavonoids may be relevant for the observed differences in biological activities as well as molecular mechanisms of action. For example, as quercetin, luteolin induces apoptosis in HL-60 cells [60]. However, luteolin acts in non-oxidative way *via* forming a luteolin-topoisomerase II-DNA ternary complex and thus mediates DNA cleavage.

In addition to modulation of cellular redox level and inhibition of topoisomerases I and II, the underlying antiproliferative activities of luteolin relate to suppression of cell survival pathways including those related to PI3K/Akt, NF- κ B and X-linked inhibitor of apoptosis protein (XIAP). It also stimulates apoptotic pathways, including those that depend on the induction of the *p53* [89, 90].

Luteolin also inhibits recombinant LSD1 *in vitro* [76]. Actually, due to the sequence homology between LSD1 and monoamine oxidases (MAO) catalytic domains, some synthetic MAO inhibitors such as *trans*-2-phenylcyclo-propylamine (TCP) and well-known natural MAO inhibitors [91,92] including luteolin, quercetin and curcumin (but not EGCG and apigenin) have been detected as potent inhibitors of LSD1 [76]. Since TCP treatment of embryonic kidney cell line HEK293 increases SIRT1 at the protein level (without recordable effect on SIRT1 mRNA level), the potent natural LSD1 inhibitors may be also expected to elevate the level of SIRT1 protein.

As measured with colorimetric HDAC Activity Assay Kit, luteolin at 50 μM inhibited total HDAC activity joined with increased acetylation of histones H3 and H4. No doubt, this finding suggests that this dietary flavonoid plays an important role in regulation of gene expression and, consequentially, has an inhibitory effect on growth of various tumor cells (LNM35, HT29, HepG2, MCF7/6 and MDA-MB231-1833) [93].

The resorcinol-like A ring was also found to be a part of flavonoid pharmacophore accounting for SIRT6 inhibition. Accordingly, luteolin inhibits deacetylase activity of recombinant SIRT 6 against H3K9 [72]. Furthermore, since luteolin is also the substrate of COMT [94], it may indirectly inhibit DNMTs by elevating endogenous SAH concentration (Figure 2) [78].

Histone H3 phosphorylation (H3S10) is a result of Aurora kinase B activity. This specific epigenetic mark is the hallmark of mitosis. Similar to quercetin [62], luteolin inhibits recombinant Aurora B enzymatic activity (equilibrium dissociation constant K_D 5.85 μM) [95]. Its inhibitory effect on endogenous Aurora B activity in different cancer cell lines was also documented [95]. The consequence of this inhibition, which was shown to be dose-dependent, is down-regulation of H3S10 phosphorylation after 24 h treatment. Inhibition of Aurora B kinase activity was also observed for fisetin and 3-hydroxyflavone [95], indicating that this effect may be related to common 2-phenyl-1,4-benzopyrone backbone (Figure 1).

In addition to inhibiting Aurora B kinase activity and hence reducing H3 phosphorylation (Figure 3), luteolin has been found to decrease the acetylation status of histone H4 at the promoter of the pro-proliferative kinase PLK-1 in PC-3 cells [88], leading to transcriptional silencing of this oncogene [83].

Luteolin is a ligand for the nuclear type II [(3H)]estradiol binding site—a binding domain on histone H4 [88]. Although, it has also been reported to stimulate c-Fos and p21 and inhibit the cell cycle pathway genes, these changes are considered to be only supportive factors in regulation of proliferation. The data obtained so far suggest that type II ligands, including luteolin, inhibit cell growth and proliferation through epigenetic control of key genes involved in cell cycle progression [88].

9. Apigenin

Apigenin is a flavone ubiquitously present in vegetables and fruits (Table 1). It induces cell cycle arrest, p53-dependent apoptosis as well as autophagy of different cancer cells. Like other polyphenols, apigenin is selectively toxic to cancer cells. However, it is much weaker radical scavenger when compared to already described flavonoids [59]. Its mechanism of action depends on cancer cell type, indicating possible existence of structural modifications caused by cellular metabolism. For example, apigenin (50 μM) blocks cellular proliferation through G2/M phase arrest of myeloid HL60 cells, leading to caspase-dependent apoptosis [96]. In contrast, it (100 μM) blocks erythroid TF1 cells in G0/G1 phase leading to autophagy. In both cell lines the JAK/STAT pathway was one of major apigenin targets.

Treatment of monocytic leukemia cells THP-1 with apigenin (50 μM) induced DNA damage mediated by kinases PKC δ and p38 [97]. The cell cycle progression at G1/S was delayed and joined with the increased number of apoptotic cells. The PKC δ -dependent phosphorylation of ataxia-telangiectasia mutated (ATM) kinase and histone γH2AX (a marker of double strand DNA breaks) upon treatment with apigenin, resulted in transcriptional down-regulation of genes involved in cell-cycle control and DNA repair. This indicates that THP-1 cells may be unable to repair DNA damage induced by apigenin, hence triggering apoptosis.

Exposure of LNCaP (hormone-dependent / p53 wild type) and PC-3 (hormone-independent / p53 mutant type) prostate cancer cells to apigenin (and also genistein, see next section) resulted in same pattern of cell cycle arrest and apoptosis, associated with p21 up-regulation and PLK1 suppression [98].

Apigenin (20-40 μM) treatment of prostate cancer cells PC-3 and 22Rv1 resulted in the inhibition of HDACs of class I, particularly HDAC1 and HDAC3, at the mRNA and protein levels [99]. Apigenin-mediated HDAC inhibition resulted in reversal of aberrant epigenetic events that promote carcinogenesis, that is, in global histone H3 and H4 acetylation, as well as localized hyperacetylation of histone H3 on the *p21* promoter. A corresponding increase in p21 and BAX mRNA and protein expressions resulted in cell cycle arrest and induction of apoptosis in both cell lines. This effect was additionally demonstrated *in vivo* by studies of PC-3 xenografts in apigenin-fed athymic nude mice.

Like already described flavonoids, arginin modulates recombinant SIRT6 deacetylase activity although more weakly than quercetin [72].

10. Genistein

Genistein is an isoflavone abundant in soybeans. Genistein represents a prototype of a phytoestrogen able to bind to nuclear hormone receptors ER- α and ER- β . It also down-regulates androgen receptor in androgen-dependent prostate cancer cell lines such as LNCaP [100]. It has been investigated as a chemopreventive agent against various cancers, particularly hormone-responsive breast and prostate malignancies. According to numerous *in vitro* experimental results accompanied with *in vivo* models, it displays pro-apoptotic, antimetastatic and antiangiogenic properties.

Genistein is only moderately strong radical scavenger [59]. However, this isoflavone exerts antioxidant effects on cellular level at low μM concentrations, which correspond to physiologically relevant concentrations in plasma. The plasma level of genistein in women consuming soy products was observed to be 0.74-6.0 μM [101]. Already at concentration of 0.5 μM , genistein increases antioxidant status of cells *via* i) interaction with estrogen receptors, ii) activation of ERK1/2 and iii) nuclear translocation of p50 subunit of NF- κB , resulting in an overexpression of MnSOD (manganese-dependent superoxide dismutase) and consequential lowering of intracellular peroxide levels [102].

Genistein can arrest cell growth and induce apoptosis in various hematological cancer cell lines [103] and cell lines of solid tumor origin (e.g. HCT-116 and SW-480 [104]). Its influence on modulation of cell cycle most commonly relates to induction of G2/M cell cycle arrest, as shown in breast, colon, malignant glioma and prostate cancer cell lines [104,105,106]. At a molecular level, this effect is commonly dependent on tumor suppressors p53, p21 and/or p16 [106].

While genistein's anticancer effects appear similar in different cancer cell lines, the underlying molecular mechanism of its action depends on a cell type. For example, genistein was shown to have a strong inhibitory effect on cellular growth followed by apoptosis, on two different prostate cancer cell lines, LNCaP-androgen-sensitive and DuPro – androgen resistant. However, it induced G0/G1 and G2/M cell cycle arrest in LNCaP and DuPro cells, respectively [107]. In addition, cellular effects of genistein on cancer cell cycle are very dependent upon applied dose [103].

Genistein has been one of the first specific protein tyrosine kinase inhibitors which was described almost 30 years ago [108,109]. As already stated, genistein antagonizes estrogen- and androgen-mediated signaling pathways [106]. It has also been found to intervene in other cellular signal transduction pathways inhibiting carcinogenesis through inhibition of NF- κ B and Akt signaling [57,109]. Significant part of genistein's action, including growth inhibition and cytotoxicity, relates to its inhibitory effect on topoisomerase II. This was very clearly shown through resistance to genistein in a model of murine transgenic cells lacking topoisomerase II beta [110,111].

Besides, genistein is also a potent modifier of epigenetic events including DNA methylation and/or histone acetylation – directly, or through steroid receptor dependent process [101]. In both aforementioned prostate cell lines, LNCaP and DuPro, genistein treatment (10-25 μ M) reshaped the chromatin structure [107]. The treatment increased acetylation level of histones H3 and H4 at the transcription start sites of the two tumor suppressor genes, *p21* and *p16*. It also increased expression of transcriptional activators HATs, but did not change the global methylation status of these cells.

Genistein's effect on breast cancer cells is selective as shown recently [112]. In a dose-dependent manner genistein inhibits both precancerous (normal human mammary epithelial cells stably transfected with SV40 and human telomerase reverse transcriptase, *hTERT*) and cancerous breast cells growth. Of importance, the apoptotic process was more prominent in precancerous than in cancerous breast cells, indicating the strong preventive potential. At the same time, there were only minor effects when applied to (commercially available) normal mammary epithelial cells [112]. *In vivo*, orthotopic breast cancer mouse model was used. The animals were orally-fed with genistein enriched diet (modified AIN-93G diet supplemented with 250 mg/kg genistein, corresponding to high-soy diet in humans). In these animals, genistein has inhibited breast cancer growth; the tumors had considerably lower wet weight and significantly less PCNA (proliferating cell nuclear antigen) positive cells, when compared with tumors that were developed in the animals fed with diet that was not supplemented with genistein. At the mRNA level, there was a strong increase in expression of tumor suppressor genes *p16* and *p21*, and dramatic decrease of oncogenes *BMI1* (polycomb complex protein BMI-1) and *c-MYC*. These changes in mRNA

expression clearly point out genistein's effect on epigenome. Indeed, this was confirmed in experiments that showed that genistein induces formation of euchromatin in the region that covers promoters of *p21* and *p16*, through changing the ratio of activating and repressive histone modifications. Specifically, genistein treatment increased histone H3 acetylation and formation of H3K4me3 and, at the same time, decreased formation of suppressive chromatin marks, H3K9me3 and H3K27me3 [112]. These changes were mild in breast cancer cells, but very prominent in precancerous breast cell line, indicating, one more time, the importance of genistein in the process of cancer prevention. Based on several scientific reports, especially in the field of breast cancer, it seems that genistein significantly influences KTMs, while its effect on HDACs does not seem to be so prominent [112].

In breast cancer cell lines MCF-7 (ER- α positive) and MDA-MB 231 (ER- α negative) genistein (18.5 μ M) induces decrease in trimethylated marks at H3K27, H3K9 and H3K4, at six selected genes: EZH2 (histone-lysine N-methyltransferase that adds methyl group to H3K27), BRCA1, ER- α , ER- β , SRC3 (steroid receptor coactivator protein 3, that displays HAT activity) and p300 [113]. Genistein treatment also affected histone acetylation marks in close proximity of these six selected genes recorded as an increase in H4K8ac and H3K4ac modifications.

In MDA-MB-231 cells, genistein (25 μ M) restored ER- α expression by remodeling the chromatin structure in the ER- α promoter [112, 114, 115]. This is very beneficial effect, as it increases tamoxifen-dependent anti-estrogen therapeutic sensitivity *in vitro* and *in vivo*. In addition, this effect was synergistically enhanced when combined with HDACi trichostatin A.

There are also experimental data on genistein's effect on colon cancer cells. When applied to HT29 cell line, genistein has been reported to have inhibitory effects on HDAC activity with IC₅₀ value of 97 ± 18 μ M [116]. Furthermore, incubation of HT29 cells with high concentration of genistein (200 μ M) resulted in a significant decrease of HDAC1 protein. Similar results were obtained on human esophageal squamous cell carcinoma cells KYSE 510, in which 5 μ M and 100 μ M genistein inhibited 13.2% and 33% of HDAC activity, respectively [101].

Genistein was also observed to decrease expression of HDACs in other types of cells, as well. In aforementioned human breast cancer cell lines, MCF-7 and MDA-MB-231, genistein treatment (15 and 10 μ M,) induced reduction (2 and ~ 4 fold, respectively) in expression of HDAC1 protein [117]. Genistein (25 μ M) has been reported to significantly downregulate expression of class II HDAC6 protein in prostate cancer cells, LNCaP [100]. Since HDAC6 is a Hsp90 deacetylase, such a change leads to an increase in the acetylated form of Hsp90 and hence inhibition of its chaperone activity. The consequence of the lack of the chaperone function is ubiquitin-mediated degradation of androgen receptor protein. However, in difference to its structural isomer apigenin (Table 1), genistein does not modulate activity of recombinant SIRT6 [72].

In addition to its impact on histone acetylation and methylation, genistein was also shown to influence DNMTs *in vitro*, at the transcriptional level and also through inhibition of DNMT1 activity [117, 118]. It reduces methylation specifically at promoters of tumor suppressor genes thus affecting cancer cell survival [101]. In KYSE 510 cells treated with 2–20 μ M genistein, the inhibition of DNMT1 activity was associated with demethylation of the CpG islands specifically in the promoters of *p16*, *RAR- β* (retinoic acid receptor beta) and *MGMT* (O6-methylgua-

nine methyltransferase) resulting in their consequential transcriptional reactivation [101,118]. Because genistein is a weak inhibitor of DNMTs, it does not induce global genomic hypomethylation but selective hypomethylation at hypermethylated region of genomic DNA, corresponding to aforementioned tumor suppressor genes, *p21* and *p16* [117].

Genistein can restore the expression of genes silenced by DNA hypermethylation in breast cancers not only by inhibiting enzymatic activity of DNMT1, but also through decrease of DNMT1 protein level [117]. Increased expression of DNMT1, DNMT3a, and DNMT3b is common in various cancer types, including breast cancer. Treatment of MCF-7 and MDA-MB-231 cell lines with genistein (as well as with other polyphenolic DNMT1 inhibitors, EGCG or curcumin), resulted in a significant decrease not only of HDAC1, but also of DNMT1 and MeCP2 protein levels, joined with a significant decrease in the transcript levels of all three DNMTs-DNMT1, DNMT3a, and DNMT3b [117]. The genistein effects on cell viability of these breast cancer cells were dose-dependent with IC_{50} values of 15 and 10 μ M for MCF-7 and MDA-MB-231 cells, respectively.

The inhibitory effects of genistein on DNMTs and HDACs may have an intrinsic synergistic effect [101]. Genistein has been observed to have additive effects on the reactivation of methylation-silenced genes in combination with other DNMT inhibitor decitabine or a HDAC inhibitor trichostatin A [101].

11. EGCG

The (-)-epigallocatechin-3-O-gallate (EGCG) is the most abundant catechin in green tea, accounting for 50-70% of green tea catechins [119]. Other green tea catechins are (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC).

EGCG is a strong free radical scavenger [59]. Its antioxidant activity is additionally enhanced by induction of expression of phase II detoxifying and antioxidant enzymes such as glutathione S-transferases (GST), glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), heme oxygenase-1 (HO-1) and many other enzymes involved in the elimination or inactivation of reactive oxygen species (ROS) and electrophiles implicated in multi-stage carcinogenesis. EGCG acts through Nrf2/ARE dependant way *via* activated Akt and ERK1/2 signaling and / or through modifying cysteine thiols of Keap1 (Kelch-like ECH-associated protein 1), a repressor of Nrf2 [120]. EGCG can also inhibit carcinogenesis by modulating some other transduction pathways including JAK/STAT, Wnt and Notch [57,121]. EGCG-induced inhibition of EGFR (epidermal growth factor receptor) pathway is well-documented. It also negatively influences topoisomerase II [122]. In majority cancer cell lines tested so far, this catechin induces G0/G1 phase cell cycle arrest. Finally, EGCG induces apoptosis through both, p53-dependent and p53-independent pathways. These activities, joined with an inhibitory effect on NF- κ B and MMPs, leads also to inhibition of angiogenesis, invansion and metastasis formation [119,121]. In addition to all these processes, chemopreventive and anticancer effects of EGCG are also based on modifications of epigenetic processes, selectively in the cancer cells. *In vitro* studies show that EGCG enters cellular nuclei and binds to both DNA and RNA [122].

As already stated, DNA methylation patterns and histone modifications significantly differ between normal and tumor cells. Accordingly, selective targeting of epigenetic marks that makes the difference between these two kinds of cells is a challenge in cancer therapy. In cancer prevention, selective "preservance" of protective epigenetic marks represents an imperative that deserves specific attention. In the field of therapy, reexpression of DNA methylation-silenced tumor suppressor genes by inhibiting the DNMTs (DNMT1, DNMT3A, and DNMT3B) has emerged as an effective strategy [123].

EGCG is a natural non-nucleoside inhibitor of DNMT1, undergoing clinical testing [124]. DNMT1 is overexpressed in the S-phase of cell cycle, particularly in highly proliferating cells, which are the basic feature of cancer. This enzyme has a high preference for hemimethylated DNA and is essential for maintaining the methylation pattern during each round of DNA replication. Strong inhibitory effect of EGCG with IC_{50} at 0.47 μ M on human DNMT1-mediated DNA methylation was independent of the COMT-mediated methylation of EGCG (Figure 2) [78]. EGCG inhibits activity of the DNMT1 molecule directly by binding to its catalytic site through formation of H-bonds [78,125,126]. This binding is stabilized by Mg^{2+} ions [78]. The galloyl moiety was shown to most considerably contribute to high-affinity of EGCG for human DNMT1. Thus, DNMT1 is also directly inhibited by other polyphenols with galloyl or also pyrogallol acid (*e.g.* myricetin) moiety [78].

In breast cancer cell lines MCF-7 and MDB-MB-231 EGCG, like genistein and also curcumin, downregulates transcription of not only DNMT1, but also *de novo* DNMTs, DNMT3a, and DNMT3b. The effect extends to the proteins, lowering the level of DNMT1, HDAC1, and MeCP2, known to interact with methylated DNA CpG regions [117]. Its effects on MCF-7 and MDB-MB-231 cell viability were dose-dependent with IC_{50} of 10 and 15 μ M, respectively.

Similarly to genistein, EGCG may, in physiologically attainable concentrations, reverse aberrant gene hypermethylation [101,118,126]. *In vitro* demethylation of specific promoters has been shown in various cancer cell types for many genes including *p16*, *MGMT*, *hMLH1* (human mutL homologue 1), *GSTP1* (Glutathione S-transferase Pi) and/or *RAR β* [118,126]. Epigenetic silencing of a phase II enzyme *GSTP1* has been recognized as a molecular hallmark of human prostate cancer. Exposure of human prostate wild-type p53 cancer cell lines, LNCaP and MDA PCa 2b, to green tea polyphenols and EGCG alone (5-20 μ M) for several days caused a concentration- and time-dependent reactivation of this enzyme, through extensive demethylation in the proximal *GSTP1* promoter and regions distal to the transcription factor binding sites. This specific action of green tea polyphenols was correlated with the inhibition of both, DNMT1 expression and activity [127]. Of importance, exposure of LNCaP cells to green tea polyphenols did not result in global hypomethylation, but, instead, promoted maintenance of genomic integrity. Additionally, the exposure of LNCaP cells to EGCG reduced transcriptional activity of genes coding for HDACs 1-3 which also resulted with their decrease at the protein levels. This molecular change was joined with the increased level of acetylated histones H3 (H3K9 and H3K18) and H4.

In the context of uniqueness of cellular epigenome, in human epidermoid carcinoma cells A431 the EGCG (5-20 μ M) treatment did decrease global DNA methylation level. Expectedly, it was joined with decrease of DNMT1 transcript, protein and enzymatic activity in a dose-dependent manner, although far less than upon the treatment with clinically approved demethylating

drug decitabine (5 μ M) [128]. In this model, EGCG treatment resulted in re-expression of the mRNA and proteins of silenced tumor suppressor genes *p16* and *p21*. There was also an increase in histones H3 (H3K9 and H3K14) and H4 (H4K5, H4K12, H4K16) acetylation and decrease in level of methylated H3K9. These phenomena were ascribed to decreased level of HDAC activity in A431 cells upon EGCG treatment [128].

In pancreatic metastatic adenocarcinoma cells AsPC-1, EGCG (10 μ M) was also shown to induce Raf kinase inhibitor protein (RKIP) *via*, in part, the inhibition of HDAC activity [129]. At the same time, this treatment repressed activation of ERK and upregulated E-cadherin expression. Histone H3 expression was also increased, while Snail (zinc finger protein SNAI1) expression, NF- κ B nuclear translocation and MMP-2 and -9 activities were inhibited. Of great importance, the treatment decreased metastatic potential of these cells.

Inhibition of HDAC activity and HDAC1 protein expression was recorded in a model of human colon carcinoma, HT29 [116]. EGCG at concentration 100 μ M, inhibited HDAC activity by about 50%. Concomitantly, a significant decrease of the HDAC1 protein level was observed depending on EGCG concentration (significant reduction at concentrations \geq 50 μ M).

EGCG mediated, dose- and time-dependent inhibition of class I HDACs (HDAC1, 2, 3 and 8) joined with the increased acetylation of histone H3 and p53, were shown in experiments that were performed on prostate cancer cell lines LNCaP and PC-3 [130]. Acetylation of p53 resulted in its binding to the promoters of *p21* and *BAX* genes leading to increased accumulation of cells in the G0/G1 cell cycle phase and induction of apoptosis. Acetylation of p53 at Lys373 and Lys382 results in p53 accumulation due to block of its MDM-2 mediated ubiquitination [130]. Finally, EGCG was suggested to contribute also to proteasomal degradation of class I HDACs [130].

Similar to quercetin, EGCG and polyphenols with the galloyl moiety in general, stimulate the activity of recombinant SIRT1 enzyme under stabilizing antioxidant conditions (achieved by adding vitamin C or catalase) [67]. Without stabilization, recombinant SIRT1 became a target for strong inhibition by these polyphenols [49], probably due to their auto-oxidation and H₂O₂ formation [67]. These data point to the importance of testing *in vitro* stability of polyphenol aglycons and tentative effects of actual products of their (bio)chemical transformations. For example, quercetin did not show any effect on the deacetylation activity of SIRT1 in HT29 cells [67].

In addition to its influence on HDACs, EGCG has been identified as a specific inhibitor of majority of HATs [131]. In HeLa cell nuclear extract, EGCG at 100 μ M concentration inhibited 90% of HAT activity while no changes in total HDAC, SIRT1 and histone methyltransferase activities were observed. The observed inhibitory action on NF- κ B activation by EGCG has been ascribed to its inhibition of RelA (p65) acetylation rather than the direct inhibition of p65 phosphorylation. The mechanism was explained using HEK293 cells in which EGCG (100 μ M) abrogated p300-induced p65 acetylation. Hypoacetylation of p65 increases the level of cytosolic I κ B α which prevents p65 translocation into the nucleus, thus interrupting the TNF α -induced cascade of events.

EGCG (50 μ M) inhibitory effect on HATs may be beneficial in hormone dependent prostate cancer due to suppression of agonist-dependent androgen receptor (AR) activation by

downregulation of its acetylation. Consequentially, AR remains "locked" in the cytoplasm and cannot activate AR-related gene transcription [132]. Like p65, androgen receptor is also acetylated by p300/CBP and PCAF (HAT KAT2B)/TIP60 (HAT KAT5), supporting the hypothesis that HAT coactivators compete with HDAC corepressors for binding to promoter regions and/or protein substrates and determine the level of transcription.

12. Curcumin

Curcumin (1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione) is a natural yellow-coloured curcumoid whose medicinal properties have long been recognized in traditional Indian Ayurvedic medicine [133]. This chemical is an important part of curry spice mix. It was brought to Western world in the 14th century.

Like other well-known polyphenols, curcumin has diverse pharmacological properties including chemopreventive activity [134]. Its proven antioxidative, anti-inflammatory, antiproliferative and antiangiogenic effects brought it in the focus for use in cancer prevention. Recently its impact on cell signal transduction pathways by regulation expression and/or activity of various proteins including NF- κ B, Akt, MAPK, p53, Nrf2, Notch-1, JAK/STAT, β -catenin, and AMPK (5'-AMP-activated protein kinase) has been reviewed [135]. Curcumin induces growth arrest at G1 or G2/M phase of cell cycle leading to apoptosis of cancer cells of various types. Indeed, if one molecule has an influence on all these processes, then it has to be that it triggers basic biochemical processes in the cell.

Pharmacokinetic analyses have shown that its concentration in human plasma is far below concentrations measured in experimental cell culture systems [136]. The most likely explanation for its observed biological activity may be related to the possibility that curcumin exerts its biological activity through remodeling the epigenome network. If this kind of action indeed takes the place, one would expect that lower concentrations are required for various biological actions.

Indeed, curcumin is a strong modulator of epigenome [137]. It reduces histone acetylation mainly *via* inhibition of HAT activity [138,139]. Its first epigenome-modifying activity was shown in 2004 through its specific inhibition of acetyltransferase activity of p300/CBP in cervical cancer cells, HeLa [138]. The consequence of this event was acetylation inhibition of histones H3 and H4 with $IC_{50} \sim 25 \mu\text{M}$. This effect was specific since the HAT activity of PCAF, histone deacetylase HDAC1 and histone methyltransferases remains unchanged. Curcumin binding to the active site of p300 also abolished p300-mediated acetylation of p53. This may be of great importance because p300/CBP directly interacts with and acetylates p53, enhances its transcriptional activation ability and, consequently, DNA repair. However, due to the fact that p53 can be target of other HATs that are not inhibited by curcumin, its acetylation status upon curcumin treatment remained in the physiological range.

Curcumin's HAT p300/CBP inhibitory activity is based on its Michael reaction acceptor functionality where its α , β unsaturated carbonyl groups in the linker (Table 1) act as reaction sites [140]. Its binding to p300/CBP induces conformational changes which result in decreased binding affinities of histones and acetyl CoA for p300 [138]. Curcumin has also been reported

to promote proteasome-dependent degradation of p300 and CBP without affecting the HATs PCAF or GCN5 [140].

HAT inhibition may be a base for a variety of pharmacological effects of curcumin. HATs, have been implicated in cancer cell growth and survival, and as such, HATs represent therapeutically relevant molecular targets for anticancer drug development. HAT inhibitors also seem promising for the treatment of Alzheimer's disease and diabetes [141]. As a co-activator, p300/CBP also enhances NF- κ B transcriptional activity by acetylating NF- κ B/p65 as well as surrounding histones. Direct inhibition and downregulation of p300/CBP therefore contribute to the inhibition of NF- κ B by curcumin. Curcumin's influence on NF- κ B activity is extremely important for its chemopreventive potency [142]. Additionally, its anti-infectious activities against HIV-1 [138] and *Plasmodium falciparum* [143] can also arise from inhibition of HAT activity.

In addition, on HeLa nuclear extracts curcumin showed the potent activity as an inhibitor of HDAC [144]. At a very high concentration of 500 μ M, curcumin was found to reduce ~ 50% HDAC activity and its IC₅₀ was 115 μ M. In the cited study, the design of the experiment did not allow for any conclusion on the mechanism of inhibition: inhibitor candidates were mixed with HeLa nuclear extract before addition of HDAC fluorometric substrate which was sensitized through deacetylation and fluorescence. Hence, only the consequence of the presence of potential inhibitor was measured. In leukemia cell lines curcumin was shown to increase expression of suppressors of cytokine signaling, SOCS1 and SOCS3, through global inhibition of HDAC activity and decreased expression of HDAC8 [145]. In curcumin (25 μ M, 24 h) treated Raji cells (Epstein-Barr virus transformed lymphocyte), a decrease of HDAC8 was joined with similar levels of decrease of HDACs 1 and 3, leading to significant increase of histone H4 acetylation [146]. Curcumin was also shown to reduce HDAC activity in medulloblastoma cells and directly inhibits transcription of HDAC4 [147]. These results represent an extension of previous scientific work showing that curcumin's free binding energy and inhibition constant for HDAC8 are comparable to trichostatin A and vorinostat [144].

Like luteolin, quercetin and resveratrol, curcumin (50 μ M) inhibits histone demethylase activity of recombinant LSD1 [76].

Curcumin can induce epigenetic modifications by modulating not only histone acetylation but also DNA methylation. It is considered to be a hypomethylating agent decreasing DNMT activity [137, 148]. Similarly to genistein and EGCG, curcumin (10 μ M) has been demonstrated to have the potential to reverse the epigenetic changes in breast cancer cells through inducing significant decrease in protein levels of DNMT1, HDAC1 and MeCP2 as well as in the transcript levels of all three DNMTs, DNMT1, DNMT3a and DNMT3b [117]. Curcumin has been found to covalently block the catalytic thiol group within catalytic site of DNMT1 with an IC₅₀ of only 3 μ M after 72 h treatment, leading to reduction of DNA methylation by 15-20%, as shown in the model of AML cell line MV4-11 [148]. Curcumin treatment decreased the mRNA and protein levels of DNMT1 and downregulated its DNA methylation activity in breast cancer cells MCF-7. The downregulation of DNMT1 mRNA may well be a consequence of lack of an NF- κ B/Sp1 binding to the promoter region of DNMT1 [149]. Both proteins are shown to bind to DNMT1 promoter and positively regulate DNMT1 expression. Thus, in curcumin treated

cells, a decrease of NF-κB and Sp1-well known curcumin targets, may directly influence the activity of DNMT1 gene.

The most recent findings indeed confirm that curcumin decreases DNMT1 expression not only in various leukemia cell lines, but also in patient’s primary AML cells treated *ex vivo* [150]. This effect was concomitant with reactivation of tumor suppressor gene *p15^{INK4B}* (p15) through hypomethylation of its promoter, G1 cell cycle arrest, and *in vitro* induction of apoptosis. This effect is, again, specific for transformed cells as it is absent in patient’s healthy PBMCs treated in this way.

In contrast to non-specific global hypomethylation induced by decitabine, curcumin treatment of three colorectal cancer cell lines HCT116, HT29 and RKO, resulted in methylation changes at selected partially-methylated loci, instead of fully-methylated CpG site [151]. Curcumin has also been found to selectively induce demethylation of promoters of *NRF2* and *RARβ2* genes [152]. It also down-regulated expression of *EZH2* and thus reduced H3K27me3 methylation, as shown in in MDA-MB-435 cells [153].

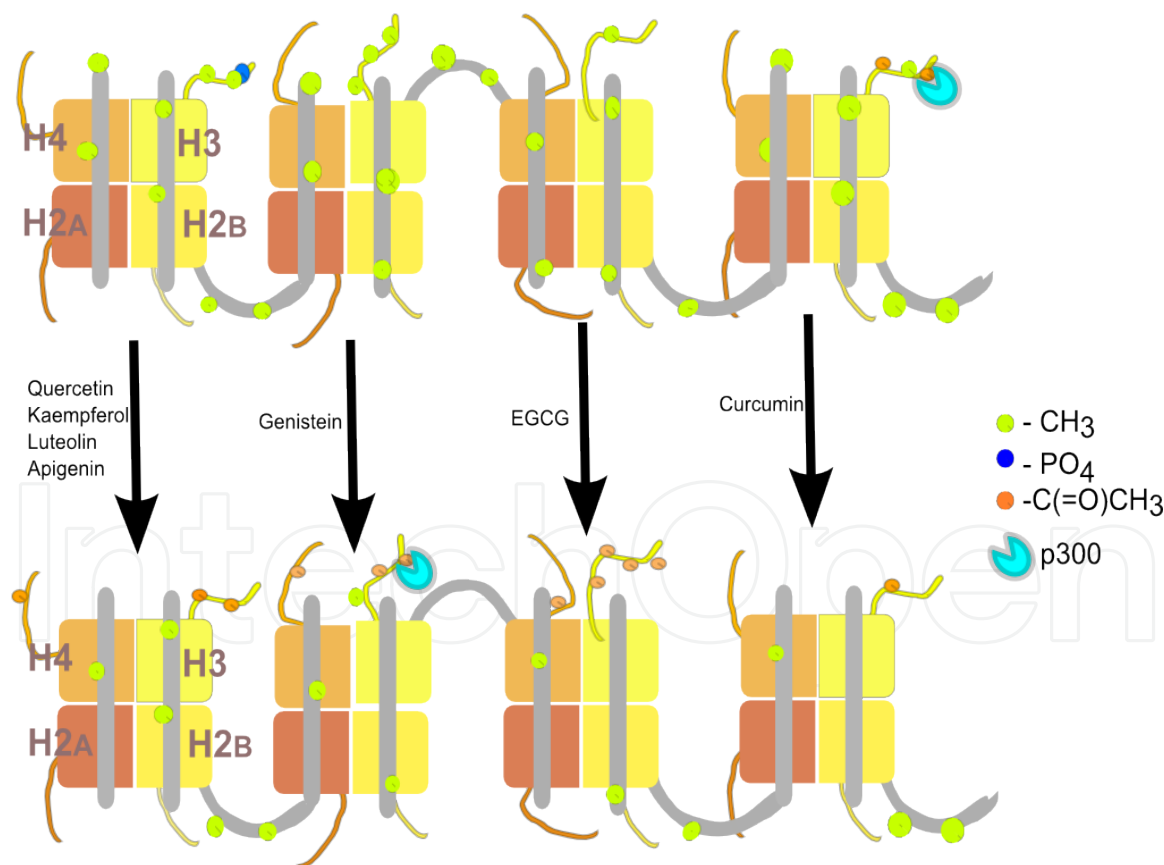


Figure 3. Epigenetic modifications induced by selected polyphenols. Molecular mechanisms of action depends upon their structures – flavonols and flavones directly inhibit histone demethylase LSD1 and indirectly DNMTs (Figure 2); genistein promotes HAT activity and DNA demethylation; EGCG is a direct DNMT inhibitor; curcumin affects many epigenome modifying enzymes.

13. Conclusion

Experimental data reported so far clearly point to dietary polyphenols as potent epigenetic modulators, able to "restore" disturbed epigenomic network which is present in premalignant and malignant cells. Polyphenols usually target more than one epigenome-modulating enzyme. In the case of curcumin the effect can be extended to enzymes with antagonistic functions. This is very interesting as it opens the question related to "epigenetic balance" and "epigenetically balanced cell". With our current knowledge, we cannot predict all possible consequences of these polyvalent modes of action.

The epigenetic effects of polyphenols are largely dependent upon compound concentration, its application time window, and, very important – on a specifically established epigenome networking which is specific for each cancer cell. As xenobiotics, natural polyphenols are substrates of various metabolic enzymes. Upon cellular uptake they can be chemically transformed by various metabolic processes that depend on both, compound concentration and the type of the cell. When studying biological effects of polyphenols, their chemical characterization has to be performed in order to detect the most probably active form.

In summary, herein considered flavonoids and curcumin represent attractive starting scaffolds for design of chemopreventive or therapeutic drugs against various cancer types. In addition, due to specific effects on epigenome, they may be useful as chemical probes that may help to understand effects of natural and synthetic molecules on epigenome and chromatin remodeling.

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